DESCRIPTION

CELLULAR DELIVERY OF NATRIURETIC PEPTIDES

Cross-Reference to Related Application

The present application claims the benefit of priority of U.S. Provisional Application Serial No. 60/319,530, filed September 6, 2002, which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

Background of Invention

Brain natriuretic peptide (BNP) is a peptide of 26 residues, with a remarkable homology to atrial natriuretic peptide (ANP). The peptide exerts natriuretic-diuretic activity as well as a potent smooth muscle relaxant activity (Sudoh *et al.*, *Biochem Biophys Res Commun*, 1988, 155:726-732). ANP administered into the rat lateral ventricle, or administered intravenously, has been reported to inhibit brain water and sodium accumulation associated with ischemic infarcts and in sub-arachnoid hemorrage-induced brain edema (Nakao *et al.*, Neurosurgery, 1990, 27:39-43; discussion 43-34; Naruse *et al.*, *Acta Neurochir Suppl (Wien)*, 1990, 51:118-121; Doczi *et al.*, *Acta Neurochir (Wien)*, 1995, 132:87-91). ANP given after a 4-hour delay significantly reduced brain water and sodium 24 hours after an experimental brain hemorrhage in rats. Neither mannitol nor 8-bromo-cGMP affected brain edema. The anti-edematous effect of ANP has been attributed to inhibition of sodium transport and the coupled water influx.

Administration of BMSC intravenously or intracerebrally has been shown to result in significant improvement in the rate of recovery from the neurological deficits produced in a rat model of stroke (Li *et al.*, *Journal of Cerebral Blood Flow & Metabolism*, 2000, 20:1311-1319; Chen *et al.*, *Stroke*, 2001, 32:1005-1011; Li *et al.*, *Neurology*, 2002, 59:514-523). Similar treatment with BMSC has also enhanced recovery from traumatic brain injury (Mahmood *et al.*, *Neurosurgery*, 2001, 49:1196-1204; Mahmood *et al.*, *Journal of Neurosurgery*, 2001, 94:589-595; Mahmood *et al.*, *J Neurotrauma*, 2002,

19:1609-1617). In all these reports, the structural repair of the brain lesion does not correlate with the recovery, leading to the hypothesis that secretion of growth factors and cytokines mediate, in part, the enhanced recovery process. Indeed, secretion of growth factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) have been demonstrated *in vitro* and *in vivo* after transplantation of BMSC (Chen *et al.*, *J Neurosci Res*, 2002, 69:687-691; Chen *et al.*, *Neuropathology*, 2002a, 22:275-279; Li *et al.*, *Neurology*, 2002, 59:514-523). Moreover, an immune-mediated response elicited by grafted bone marrow cells may play a role in recovery (Li *et al.*, *Neurology*, 2002, 59:514-523).

The present inventors have discovered a new way to deliver anti-edema agents utilizing a cellular vehicle that generates natural anti-edemic agents, the natriuretic peptides. In light of the beneficial effects of intravenous administration or intracerebral grafting of BMSC in animal models of stroke and brain trauma, it is strongly suggested that the enhanced recovery from neurologic deficits is mediated in part by BNP secreted by BMSC *in situ*.

The natriuretic peptides have as one of their physiological functions the ability to increase excretion of sodium and water by the kidney and to pump sodium and water from individual cells. ANP are produced in great amounts in the heart, especially during heart failure. BNP is found in the hypothalamus of the brain, but is also found at much higher concentrations in heart tissue. These peptides have been shown to be effective when delivered directly into brain tissue (through an indwelling probe), but until the present inventors' discovery, these peptides could not be delivered systemically because of rapid proteolysis in the system circulation.

Brief Summary of Invention

The present invention pertains to a method for treating deficits caused by focal or generalized edema associated with injury to organs or organ systems, such as the central nervous system, heart, liver, and kidneys. According to the method of the present invention, edema associated deficits are treated by the administration of cells that produce natriuretic peptides, such as bone marrow stromal cells. Where bone marrow stromal

cells are utilized, the cells are preferably conditioned with retinoic acid and nerve growth factor before, during, or after administration to the patient, in order to increase the cells' production of natriuretic peptide. The method of the present invention can be used to treat neurological deficits of the central nervous system that result from stroke, trauma, toxins, and other nervous system insults.

In another aspect, the present invention concerns pharmaceutical compositions comprising bone marrow stromal cells and effective amounts of retinoic acid and nerve growth factor to induce the bone marrow stromal cells to increase production of natriuretic peptides.

In another aspect, the present invention provides a method for producing natriuretic peptides comprising culturing bone marrow stromal cells and isolating the natriuretic peptides from the bone marrow stromal cells. Preferably, the bone marrow stromal cells are cultured in the presence of retinoic acid and nerve growth factor, thereby inducing the bone marrow stromal cells to increase production of the natriuretic peptides.

In another aspect, the present invention pertains to cells that have been genetically modified to produce a natriuretic peptide.

Brief Description of Drawings

Figure 1 shows micrographs of bone marrow stromal cells (BMSCs) cultured in the presence or absence of growth factors. BMSCs were cultured under two conditions depicted in the rows A (Dulbecco Modified Eagle's Medium and fetal bovine serum (DMEM+FBS) for 6 days) and B (DMEM+FBS for 2 days, then retinoic acid and nerve growth factor (RA+NGF) for 4 days). Each row depicts the same visual field viewed with phase contrast microscopy (left frame), and fluorescence microscopy (middle and right frames). The column on the left reveals the morphological change from flat large epithelioid cells to fibroblastic morphology induced by adding RA+NGF (phase microscopy). The middle column shows BNP immunoreactive cells. The top row (condition A) illustrates several large cells (arrows) that do not show BNP immunoreactivity whereas all the fibroblastic cells in Condition B are BNP immunoreactive. The third column reveals DAPI-stained nuclei. (scale bar=50 μm).

Figure 2 shows results from a single reverse transcriptase-polymerase chain reaction (PCR) experiment run to confirm the presence of brain natriuretic peptide in BMSCs. Figure 2: Lanes 1, 3, and 5 were from cultures incubated in DMEM+FCS. Lanes 2, 4 and 6 were from cultures incubated in RA+NGF. Msh1 and nestin are normally expressed by neural progenitors and are also detected in the BMSC under both conditions of culture; BNP= brain natriuretic peptide; Msh1=musashi-1.

Figure 3 shows results obtained by applying real-time PCR to RNA extracted from BMSC cultures. Figure 3: RNA samples were extracted from each of four cultures prepared from two different donor sources. Conditions A and B were used in cells from one donor and conditions A and C were used in cells from a different donor. The RNA was reversed transcribed into cDNA and real-time PCR was carried out as described in the methods section. Amounts of human BNP and 18s RNAs were calculated using linear regression analysis from an external standard curve. Data is plotted as BNP mRNA – 18s RNAs on the Y-axis and culture conditions on the X-axis.

Figure 4 shows summary data using BMSCs from four different marrow samples. Figure 4 2-way ANOVA demonstrated that culture conditions as well as cell/supernatant contributed significantly to the variance ($p < 0.05$ for culture condition and $p<0.001$ for supernatant). t-tests with Bonferroni correction show significant differences between cells and supernatant for condition B* ($p < 0.001$) and for condition C **($p < 0.01$) but not for condition A ($p>0.05$).

Brief Description of Sequences

SEQ ID NO:1 is the gene encoding human brain natriuretic peptide (BNP) (NCBI ACCESSION # M31776; Seilhamer, J.J. *et al.*, *Biochem. Biophys. Res. Commun.*, 165(2):650-658, 1989):

1 ctgtgagatc accccgtgct cccagcgctc acgtcggtcc tcggaaagcc ggggtcctcc
61 ctgccttttc cagcaacggt ggggtgggga ggcaggaaga aagcgccaac ctaggacccc
121 ggagatttgc agcaaaggaa gaagcgggag acgggcactt gtctgtgtct ccagcgcgtt
181 cctgcccccc gccgacccgg cccatttcta tacaaggtcg ctctgcccgg tctccacctc
241 ccacgtgcag gccgcggagg ggctcattcc cgggccctga tctcagaggc ccggaatgtg
301 gctgataaat cagagactag acctgcatgg caggcaggcc cgacactcag ctccaggata

361 aaaggccacg gtgtcccgag gagccaggag gagcaccccg caggctgagg gcaggtggga
421 agcaaacccg gacgcatcgc agcagcagca gcagcagcag aagcagcagc agcagcctcc
481 gcagtccctc cagagacatg gatccccaga cagcaccttc ccgggcgctc ctgctcctgc
541 tcttcttgca tctggctttc ctgggaggtc gttcccaccc gctgggcagc cccggttcag
601 cctcggactt ggaaacgtcc gggttacagg tgagagcgga gggcagctca gggggattgg
661 acagcagcaa tgaaagggtc ctcacctgct gtcccaagag gccctcatct ttcctttgga
721 attagtgata aaggaatcag aaaatggaga gactgggtgc cctgaccctg tacccaaggc
781 agtcggttca cttgggtgcc atgaagggct ggtgagccag gggtgggtcc ctgaggcttg
841 gacgccccca ttcattgcag gagcagcgca accatttgca gggcaaactg tcggagctgc
901 aggtggagca gacatccctg gagcccctcc aggagagccc ccgtcccaca ggtgtctgga
961 agtcccggga ggtagccacc gagggcatcc gtgggcaccg caaaatggtc ctctacaccc
1021 tgcgggcacc acgaagcccc aagatggtgc aagggtctgg ctgctttggg aggaagatgg
1081 accggatcag ctcctccagt ggcctgggct gcaaaggtaa gcacccccctg ccaccccggc
1141 cgccttcccc cattccagtg tgtgacactg ttagagtcac tttggggttt gttgtctctg
1201 ggaaccacac tctttgagaa aaggtcacct ggacatcgct tcctcttgtt aacagccttc
1261 agggccaagg ggtgcctttg tggaattagt aaatgtgggc ttatttcatt accatgccca
1321 caataccttc tccccacctc ctacttctta tcaaaggggc agaatctcct ttgggggtct
1381 gtttatcatt tggcagcccc ccagtggtgc agaaagagaa ccaaacattt cctcctggtt
1441 tcctctaaac tgtctatagt ctcaaaggca gagagcagga tcaccagagc aatgataatc
1501 cccaatttac agatgaggaa actgaggctc agagagttgc attaagcctc aaacgtctga
1561 tgactaacag ggtggtgggt ggcacacgat gaggtaagct cagcccctgc ctccatctcc
1621 caccctaacc atcatcaccc tctctctttc cctgacagtg ctgaggcggc attaagagga
1681 agtcctggct gcagacacct gcttctgatt ccacaagggg ctttttcctc aaccctgtgg
1741 ccgcctttga agtgactcat ttttttaat gtatttatgt atttatttga ttgttttata
1801 taagatggtt tcttaccttt gagcacaaaa tttccacggt gaaataaagt caacattata
1861 agctttatct tttgaaactg atttgtcttg gcgcattaaa aataatccct catttcaaag
1921 aa

SEQ ID NO:2 is the amino acid sequence of human BNP:

MDPQTAPSRALLLLLFLHLAFLGGRSHPLGSPGSASDLETSGLQEQRNHLQGKLS
ELQVEQTSLEPLQESPRPTGVWKSREVATEGIRGHRKMVLYTLRAPRSPKMVQG
SGCFGRKMDRISSSSGLGCKVLRRH.

SEQ ID NO:3 is the gene encoding human atrial natriuretic peptide (ANP) (NCBI ACCESSION # NM_006172; Zivin, R.A. *et al.*, *Proc. Natl. Acad. Sci. USA*, 81(20):6325-6329, 1984):

1 tggcgaggga cagacgtagg ccaagagagg ggaaccagag aggaaccaga ggggagagac
61 agagcagcaa gcagtggatt gctccttgac gacgccagca tgagctcctt ctccaccacc
121 accgtgagct tcctcctttt actggcattc cagctcctag gtcagaccag agctaatccc
181 atgtacaatg ccgtgtccaa cgcagacctg atggatttca agaatttgct ggaccatttg
241 gaagaaaaga tgcctttaga agatgaggtc gtgcccccac aagtgctcag tgagccgaat
301 gaagaagcgg gggctgctct cagccccctc cctgaggtgc ctccctggac cggggaagtc
361 agcccagccc agagagatgg aggtgccctc gggcggggcc cctgggactc ctctgatcga
421 tctgccctcc taaaaagcaa gctgagggcg ctgctcactg cccctcggag cctgcggaga
481 tccagctgct tcgggggcag gatggacagg attggagccc agagcggact gggctgtaac
541 agcttccggt actgaagata acagccaggg aggacaagca gggctgggcc tagggacaga
601 ctgcaagagg ctcctgtccc ctggggtctc tgctgcattt gtgtcatctt gttgccatgg
661 agttgtgatc atcccatcta agctgcagct tcctgtcaac acttctcaca tcttatgcta
721 actgtagata aagtggtttg atggtgactt cctcgcctct cccaccccat gcattaaatt
781 ttaaggtaga acctcacctg ttactgaaag tggtttgaaa gtgaataaac ttcagcacca
841 tggac

SEQ ID NO:4 is the amino acid sequence of human ANP:

MSSFSTTTVSFLLLLAFQLLGQTRANPMYNAVSNADLMDFKNLLDHLEEKMPLE
DEVVPPQVLSEPNEEAGAALSPLPEVPPWTGEVSPAQRDGGALGRGPWDSSDRS
ALLKSKLRALLTAPRSLRRSSCFGGRMDRIGAQSGLGCNSFRY.

SEQ ID NO:5 is the gene encoding dog (Canine) brain natriuretic peptide (BNP) (NCBI ACCESSION # M31777; Seilhamer, J.J. *et al.*, *Biochem. Biophys. Res. Commun.* 165(2):650-658, 1989):

1 cgatcaggga tgttggggcg gaggaaacgg agggaaggag ggagcggagg aggcccgagg
61 actgttggtg tccccctcct gccctttgg ggccaggccc acttctatac aaggcctgct
121 ctccagcctc caccccggcg ggtatggtgc aggcgcggag gggcgcattc ccccgccctg
181 agctcagcgg ccggaatgcg gccgataaat cagagataac cccaggcgcg ggataaggga
241 taaaaagccc ccgttgccgc gggatccagg agagcacccg cgccccaagc ggtgacactc
301 gaccccggtc gcagcgcagc agctcagcag ccggacgtct ctttccccac ttctctccag
361 cgacatggag ccctgcgcag cgctgccccg ggccctcctg ctcctcctgt tcttgcacct
421 gtcgccactc ggaggccgcc cccacccgct gggcggccgc agccccgcct cggaagcctc
481 ggaagcctca gaagcctcgg ggttgtgggc cgtgcaggtg agcgctcagc ctgcctgaag
541 gccgcggcgg gtggcagcag gtcacggggg cttagccact gtcccaagtc ctcagtctcc
601 cttgggaatt agtgataagg gaatcagaaa gtgacgagat tgggtgccag gactccatac
661 ccaaggcggc ggcttcactt gggtgcaagg gtggttccgc cccggcgtgg gttcctgagg
721 ctcaggccgt ccattgcagg agctgctggg ccgtctgaag gacgcagttt cagagctgca
781 ggcagagcag ttggccctgg aacccctgca ccggagccac agccccgcag aagccccgga
841 ggccggagga acgccccgtg gggtccttgc accccatgac agtgtcctcc aggccctgag
901 aagactacgc agccccaaga tgatgcacaa gtcagggtgc tttggccgga ggctggaccg
961 gatcggctcc ctcagtggcc tgggctgcaa tggtaagccg cctccctgcc gccttggctc
1021 cccctcccca gccccctggg ttcgaccctt ggaacccctt ctgggtttgt tgtctcgggg
1081 gatcacactc tgaggaaagg acatctggac atcgctcctt cttgctgaca gtcctaaggg
1141 ccaaggagta cgtttctgga aatactacgt gtggacatcg ttgtccaggg tccctaccca
1201 cctcctagcc ccctcctgcc tctcgcaccc aagggcagaa tcatcttagg atggaatcag
1261 tcgttgtctg gaagcatctc cttggagcag aaagagtcct aaacatcgtc ctcgtagctc
1321 tctctgtctg tctgtagcca cgaaggcaga ggtcagggtc accagggcag tgatgattcc
1381 cagttaacag aggaggagac tgaggtctag agagatggat tattccaaag cctcaaacat
1441 ccagatcggc tgagggtggg gttggtggca gggatggctc ctgggcttgg gaagctcgga
1501 tcctgcctca gtctcccacc tgacgccatc atccccctct ctctcctccc acagtgctga
1561 gaaagtatta aggaggaagt cccgactgcc cacatctgca ttggattctt cagcagcccc
1621 tgagcccctt ggaagcagat cttatttatt cgtatttatt tatttattta tttcgattgt
1681 tttatataag atgatcctga cgcccgagca cggattttcc acggtgaaat aaagtcaacc
1741 ttagagcttc ttttgaaacc gatttgtccc tgtgcattaa aagtaacaca tcatttaaaa
1801 aaa

SEQ ID NO:6 is the amino acid sequence of dog (Canine) BNP:
MEPCAALPRALLLLLFLHLSPLGGRPHPLGGRSPASEASEASEASGLWAVQELLG
RLKDAVSELQAEQLALEPLHRSHSPAEAPEAGGTPRGVLAPHDSVLQALRRLRSP
KMMHKSGCFGRRLDRIGSLSGLGCNVLRKY.

SEQ ID NO:7 is the hBNP-exon M2 probe.

SEQ ID NO:8 is a forward primer for human BNP.

SEQ ID NO:9 is a reverse primer for human BNP.

SEQ ID NO:10 is the nucleotide sequence encoding human BNP (NCBI ACCESSION # M31776):

1 atggatcccc agacagcacc ttcccgggcg ctcctgctcc tgctcttctt gcatctggct
61 ttcctgggag gtcgttccca cccgctgggc agccccggtt cagcctcgga cttggaaacg
121 tccgggttac aggagcagcg caaccatttg cagggcaaac tgtcggagct gcaggtggag
181 cagacatccc tggagcccct ccaggagagc ccccgtccca caggtgtctg gaagtcccgg
241 gaggtagcca ccgagggcat ccgtgggcac cgcaaaatgg tcctctacac cctgcgggca
301 ccacgaagcc ccaagatggt gcaagggtct ggctgctttg ggaggaagat ggaccggatc
361 agctcctcca gtggcctggg ctgcaaagtg ctgaggcggc attaa

SEQ ID NO: 11 is the nucleotide sequence encoding human ANP (NCBI ACCESSION # NM_006172):

1 atgagctcct tctccaccac caccgtgagc ttcctccttt tactggcatt ccagctccta
61 ggtcagacca gagctaatcc catgtacaat gccgtgtcca acgcagacct gatggatttc
121 aagaatttgc tggaccattt ggaagaaaag atgcctttag aagatgaggt cgtgccccca
181 caagtgctca gtgagccgaa tgaagaagcg ggggctgctc tcagcccccc ccctgaggtg
241 cctccctgga ccggggaagt cagcccagcc cagagagatg gaggtgccct cgggcggggc
301 ccctgggact cctctgatcg atctgccctc ctaaaaagca agctgagggc gctgctcact
361 gcccctcgga gcctgcggag atccagctgc ttcgggggca ggatggacag gattggagcc

421 cagagcggac tgggctgtaa cagcttccgg tactga

SEQ ID NO:12 is the mouse type-B natriuretic peptide gene (NCBI ACCESSION # S58667; Steinhelper,M.E., *Circ. Res.* 72(5): 984-992, 1993; coding sequences (CDS) = join nucleotides 211-336,531-753,1188-1204):

1 gaattctcag gtcctgagct cagccggcag gaatgcagct gataaatcag agataacccc
61 acccctactc cgtgaaaagg tctggccgga cactcagccc cagtataaaa ggcagaggca
121 ccgttgttga agacaccagt gcacaagctg cttggggagg cgagacaagg gagaacacgg
181 catcattgcc tggcccatcg cttctgcggc atggatctcc tgaaggtgct gtcccagatg
241 attctgtttc tgcttttcct ttatctgtca ccgctgggag gtcactccca tcctctggga
301 agtcctagcc agtctccaga gcaattcaag atgcaggtga gcactgaggg tctgcctgaa
361 gggtttggga agcggcaatg aaaagacctc gagtcctttg ggaattagcc atgtgagagt
421 cagcaaagtg aaagattggg cagcatatct cttaactgat gagcactatg gaaggatggg
481 ggattcaggt gtgtgtgttt ctgacgtctg ggctccccaa tccatcacag aagctgctgg
541 agctgataag agaaaagtca gaggaaatgg cccagagaca gctcttgaag gaccaaggcc
601 tcacaaaaga acacctaaaa agagtccttc ggtctcaagg cagcaccctc cgggtccagc
661 agagacctca aaattccaag gtgacacata tctcaagctg ctttgggcac aagatagacc
721 ggatcggatc cgtcagtcgt ttgggctgta acggtgagca cctaccttgc cacttccctg
781 caaagctgca ccacccatcc catccccgtg catgctaccc ttagaggccc ctaggtttgc
841 tatctggcat actcctgcag cctgtcagga aatatcacat gggttctgca ttacattctc
901 acaggtcagc acctaccttc catcagaggg tcacacgctc tgaggagcag actgctgatg
961 tctatcaccc cttcacaagg cagaaagagt ctgagcattc ccctcaggca aagggcatgc
1021 ccaacccact ttacaggaga aacagaggcc ctctgagata gctttttcca gagccttaaa
1081 cttcgacatc atctggggac tgaagatggg ggtgtggtgg tggtgggggа ctcggcacct
1141 gcttcagttt cacttcgagt gtgacattgc ctgtctctcc tccacagcac tgaagttgtt
1201 gtaggaagac ctccttggct gcaggagagc tccagtttct gactctgccg ggtctctttc
1261 cctagctctg gaccacctct gaagtgatcc tatttattta tttatttata tttattattt
1321 atttttattt ttatttttta atttaatttt gttgtttttc acagctgttg ttacttggag
1381 cacaaactgc cacaacataa taaacatacg ttatttcctg cttttgaaaa gg

SEQ ID NO:13 is the mouse BNP gene for natriuretic peptide (NCBI ACCESSION # D16497; Ogawa, Y. *et al.*, *J. Clin. Invest.*, 93(5):1911-1921, 1994; coding sequences (CDS): join nucleotides 210-335, 530-752, 1196-1212):

1 gaattctcag gtcctgagct cagccggcag gaatcagctg ataaatcaga gataacccca

61 cccctactcc gtgaaaaggt ctggccggac actcagcccc agtataaaag gcagaggcac

121 cgttgttgaa gacaccagtg cacaagctgc ttggggaggc gagacaaggg agaacacggc

181 atcattgcct ggcccatcgc ttctgcggca tggatctcct gaaggtgctg tcccagatga

241 ttctgtttct gcttttcctt tatctgtcac cgctgggagg tcactcctat cctctgggaa

301 gtcctagcca gtctccagag caattcaaga tgcaggtgag cactgagggt ctgcctgaag

361 ggtttgggaa gcggcaatga aaagacctcg agtcctttgg gaattagcca tgtgagagtc

421 agcaaactga aagattgggc agcatatctc ttaactgatg agcactatgg aaggatgggg

481 gattcaggtg tgtgtgtttc tgacgtctgg gctccccaat ccatcacaga agctgctgga

541 gctgataaga gaaaagtcgg aggaaatggc ccagagacag ctcttgaagg accaaggcct

601 cacaaaagaa cacccaaaaa gagtccttcg gtctcaaggc agcaccctcc gggtccagca

661 gagacctcaa aattccaagg tgacacatat ctcaagctgc tttgggcaca agatagaccg

721 gatcggatcc gtcagtcgtt tgggctgtaa cggtgagcac ctaccttgcc acttccctgc

781 aaagctgcac acccatccca tccccgtgca tgctaccctt agaggcccct aggtttgcta

841 tctggcatac tcctgcagcc tgtcaggaaa tatcacatgg gttctgcatt acattctcac

901 aggtcagcac ctaccttcca tcagaggggt cacacgctct gagggagcag actgcctgat

961 gtctaatcac cccttcacaa ggcagaaaga gttctgagca tttcccctca ggcaaagggc

1021 atgcccaacc cactttacag gagaaacaga ggccctgtga gatagctttt tccagagcct

1081 taaacttcga catcatctgg ggactgaaga tgggggtgtg gtggtggtgg gggactcggc

1141 acctgcttca gtttcacttc cgagtgtgac attgccctgt ctctcctccc cacagcactg

1201 aagttgttgt aggaagacct cctggctgca ggagactcca gtttctgact ctgcctgggt

1261 ctctttcccc agctctggga ccacctttga agtgatccta tttatttatt tatttatatt

1321 tatttttatt tttatttttt aatttatttt gttgtttttc tacaagactg tttcttatct

1381 tggagcacaa acttgccaca acataataaa catagcgtat ttcctgcttt tgaaaaggat

1441 ttgtgtccgt gagtttcaat ctatctct

SEQ ID NO:14 is the rat BNP mRNA (NCBI ACCESSION # M25297; Kojima M. *et al.*, *Biochem. Biophys. Res. Commun.*, 159(3):1420-1426, 1989; coding sequence (CDS): join nucleotides 58-423):

1 gcgagacaag agagagcagg acaccatcgc agctgcctgg cccatcactt ctgcagcatg
61 gatctccaga aggtgctgcc ccagatgatt ctgctcctgc ttttccttaa tctgtcgccg
121 ctgggaggtc actcccatcc cctgggaagt cctagccagt ctccagaaca atccacgatg
181 cagaagctgc tggagctgat aagagaaaag tcagaggaaa tggctcagag acagctctca
241 aaggaccaag gccctacaaa agaacttcta aaaagagtcc ttaggtctca agacagcgcc
301 ttccggatcc aggagagact tcgaaattcc aagatggcac atagttcaag ctgctttggg
361 cagaagatag accggatcgg cgcagtcagt cgcttgggct gtgacgggct gaggttgttt
421 taggaagacc tcctggctgc agactccggc ttctgactct gcctgcggct cttctttccc
481 cagctctggg accacctctc aagtgatcct gtttatttat ttgtttattt atttatttt
541 atgttgctga ttttctacaa gactgtttct tatcttccag cacaaacttg ccacagtgta
601 ataaacatag cctatttctt gcttttgg

SEQ ID NO:15 is the porcine BNP mRNA (NCBI ACCESSION # M25547; Porter *et al.*, *J. Biol. Chem.*, 264(12):6689-6692, 1989; coding sequence (CDS): join nucleotides 94-216, 463-718, 1273-1289):

1 caggctgcta ggaagtgaaa agtgaacctg gacccagctc agcggcagca gcagcggcag
61 caggcagcag cctctatcct ctcctccagc cacatgggcc cccggatggc gcttccccgc
121 gtgctcctgc tcctgttctt gcacctgttg ctgctaggat gccgttccca tccactgggt
181 ggcgctggcc tggcctcaga actgccaggg atacaggtga gccctgatga actgcttaga
241 cttggttggc tgggagggcg cggacagcag caactaacgg gtccccacct actgttccaa
301 gagggctcta acctcctttg ggaactagtg ataaggggtt agaaggcagc caggctgggg
361 gtgaggaccc cgctcccaag gcagttggtt cgcttcagca ccatcaagag tgatgggtcc
421 aggtgcgagt tcctgaggct cgggctcccc cacccatccc aggagctgct ggaccgcctg
481 cgagacaggg tctccgagct gcaggcggag cggacggacc tggagccccc ccggcaggac
541 cgtggcctca cagaagcctg ggaggcgagg gaagcagccc ccacgggggt tcttgggccc
601 cgcagtagca tcttccaagt cctccgggga atacgcagcc ccaagacgat gcgtgactct
661 ggctgctttg ggcggaggct ggaccggatc ggctccctca gcggcctggg ctgcaatggt
721 gagcacccac ccccattccc actgcacgcc ccggttagca tcacttctgg gtttgatgtc

781 tctggggacc aaactccgag aaaaggacac ctggatatca ctctttcttg ttgccagtcc
841 tcaaggccaa ggagcgcctt cctggaaaaa ttaaatttgg acagcattca ctagcatgac
901 tatgagtccc cacccacctt ctcgccaccc cctgcctctc tcacccaagg cggcagaatt
961 actttaggat gtaaattctg tcattgcctg gctgccgctc ctgggagcaa aaagagaact
1021 aaacctcttc cccctggttt cccctcaact gtctgtggct gcaaaggcag agggcaggat
1081 caccagggtg atgacaagtc ccagcttaca aggaggaaac tcaggtccag agagatggat
1141 tatcccaaag ccccaaacat ccagttctgc tgaagaaggc gggtggcagg ggtggcacgt
1201 ggtgggggga agcccaggtc ctgcctgcct ctcaccctaa tgtcatcctc accctctctc
1261 tccccccccac agtgctcagg aggtactgag aagtcctggc tgacaacctc tgtgtccgct
1321 tctccaacgc ccctcccctg ctccccttca aagcaactcc tgtttttatt tatgtattta
1381 tttatttatt tatttggtgg ttgtatataa gacggttctt atttgtgagc acatttttc
1441 catggtgaaa taaagtcaac attagagctc tgtcttttg

SEQ ID NO:16 is the bovine ANP gene (NCBI ACCESSION # M13145; Vlasuk, G.P. *et al.*, *Biochem. Biophys. Res. Commun.*, 136(1):396-403, 1986; coding sequence (CDS): join nucleotides 313-432, 531-857, 1383-1394):

1 ctgcagctga gggtcctggg ggttgtcggg gctgctcaag gcagaggggc tgtgacaagc
61 aggctggact gataacttta aaagggcatc ttctgctgct tcctcactca gctgctttat
121 cactgcaagt gacagaatgg ggagggttcc gtccctctcc cggacgagct cccagagagc
181 caggggggcta taaaaagagg aggctcaggg cagctgggag acagagacgg acaaaggcca
241 acagcaaaag gccaaagagg acagggagga ggcagcaagc accagaccga ccattccttg
301 accgacgcca gcatgggctc ctccgccatc accgtgagct tcctcctctt tctggcattt
361 cagctcccag ggcaaacagg agcaaatccc gtgtatggct ctgtgtccaa tgcagacctg
421 atggatttca aggtagggcc agggaacggc gatggtctgg ggctgagggg gttgtgacat
481 tgtgccaggc gagcgagacc tctccctttc cctgttttcc ttttgtaaag aatttgctgg
541 accgtttgga ggacaagatg cctttagaag atgaggctgt gccctcacaa gtactaagtg
601 agcagaatga agaagctggg gcccctctca gccccctttc agagatgcct ccctggatgg
661 gggaggtcaa cccagcccag agagaggggg gcgtcctcgg gcggggcccc tgggaatcct
721 ccgatagatc tgccctcctg aagagcaagc tgagggcact gctcactgcc cctcggagcc
781 tgcggaggtc cagctgcttc gggggaagga tggacaggat tggagcccag agtggattgg
841 gctgcaacag cttccgggta agaggacctg agaatggaaa tgggatgggg aggaaggaaa

901 ttgtggcttc attgaagttc aaaccttgtg aaagaacatc gccagggaat gccttcagta
961 ggaaagggac agcatagaag caaccccttt gaaatttctg ccccaacttg gcagggagga
1021 gggtgtgctc tgagtctcag gacaatgata ccaacctagc tacagttttc tgagagaatg
1081 ctaagaaaaa aagactttac tgccacgagc actggggact taaattgttc atggggccaa
1141 ataacctgtg ctttgctgat tggtagtttg tgtcctttgc agaatcatca gatcccaaag
1201 gattgaaatt gagcaggact gactttacta gttcctaatg ggcaatttgt ttaccagttt
1261 atagaagtca gagggtcatc aggctggagt ggaggctggt gggaagggag cacagtctga
1321 tgaagctggc ttttccagtg gagtcaggtc accaaaccaa acatgtctct gctctcttgt
1381 agtatcgaag ataatggcca gggaggaaaa ggcaggccag gccctgggca gtcttcaaga
1441 gaatcccctg gggtctctca ctcaactttg tcgcatctgg ttgccatcaa gttgagctgt
1501 gaccgagcat tcaagcatca gcttcttgtc aacatttctc acattttatg ctaaatgtag
1561 acaaagtgat ttaactgtgg ccttctccac ctctcccacc catgtgttaa gttttaatca
1621 cctgttacca acatcagttt gaaaatgaat aaacttcagc accatggaca gaagcagtag
1681 gctcgggttg gtgtgatttc tttcatttcc ggaagggagt tcagcctgat actccttgtc
1741 attttacctt ttgttggaga gaagaattc

SEQ ID NO: 17 is the rat iso-ANP gene (NCBI ACCESSION # M60731; Roy, R.N. and Flynn, T.G., *Biochem. Biophys. Res. Commun.*, 171(1):416-423, 1990; coding sequence (CDS): join nucleotides 58-183, 405-627, 1080-1096):

1 gcgagacaag agagagcagg acaccatcgc agctgcctgg cccatcactt ctgcagcatg
61 gatctccaga aggtgctgcc ccagatgatt ctgctcctgg ttttccttaa tctgtcgccg
121 ctgggaggtc actcccatcc cctgggaagt cctagccagt ctccagaaca atccacgatg
181 caggtgagca ccgagggtct gcatagggag atggagcctg cctgaagggt tttgggcagc
241 agcaatgaaa agacctcatg tcctttggga attaaccacg cgagagtcag gaaacggaaa
301 gattgggcag cagatccctt aaccacaggc actgtggaag ggtgggggag ccaggtgtgt
361 atgtgtgtgt gtgtctgagg tctgggcttc ccaattcgtc acagaagctg ctggagctga
421 taagagaaaa gtcagaggaa atggctcaga gacagctctc aaaggaccaa ggccctacaa
481 aagaacttct aaaaagagtc cttaggtctc aagacagcgc cttccggatc caggagagac
541 ttcgaaattc caagatggca catagttcaa gctgctttgg gcagaagata gaccggatcg
601 gcgcagtcag tcgcttgggc tgtgacggtg agcacctacc ttgccgcttc cctgcaaagc
661 tgcacgcatc ccgttcccct gcatgccgcc ctcagaggcc ccttggtttg ctctcagaca

721 tacttgcaca gcctgcctct accttaccga cagtcttcaa gaccaaggca gtctgtcagg
781 aagtctcaca tgggtacttc attacaccgt cccaggtgag cacctacctc cttcagaggt
841 gtcacaggct tcccagggaa cagactgcct gatgtctgat cactctgagc atctcccctc
901 cgtcttcacc aaactgaatt atccgaggca aagggcaggc ccagtgagat agcttttccc
961 agagccgtta aacttcgaca tcatctggga accaaagatg ggggtgcggt gtggcagggg
1021 aagctcagct cctgcctcag tttcactccc cagtctgaca ctggtttctc ctcccacagg
1081 gctgaggttg ttttaggaag acctcctggc tgcagactcc ggcttctgac tctgcctgcg
1141 gctcttcttt ccccagctct gggaccacct ctcaagtgat cctgtttatt tatttgttta
1201 tttatttatt tttatgttgc tgattttcta caagactgtt tcttatcttc cagcacaaac
1261 ttgccacagt gtaataaaca tagcctattt cttgcttttg g

SEQ ID NO:18 is the amino acid sequence of bovine natriuretic peptide:

1 mgssaitvsf llflafqlpg qtganpvygs vsnadlmdfk nlldrledkm pledeavpsq
61 vlseqneeag aplsplsemp pwmgevnpaq reggvlgrgp wessdrsall ksklrallta
121 prslrrsscf ggrmdrigaq sglgcnsfry rr.

SEQ ID NO:19 is the amino acid sequence of mouse BNP:

1 mdllkvlsqm ilfllflyls plgghsyplg spsqspeqfk mqkllelire kseemaqrql
61 lkdqgltkeh pkrvlrsqgs tlrvqqrpqn skvthisscf ghkidrigsv srlgcnalkl
121 l

SEQ ID NO: 20 is the amino acid sequence of rat BNP:

1 mdlqkvlpqm illlflnls plgghshplg spsqspeqst mqkllelire kseemaqrql
61 skdqgptkel lkrvlrsqds afriqerlrn skmahssscf gqkidrigav srlgcdglrl
121 f.

SEQ ID NO:21 is the amino acid sequence of porcine BNP:

1 mgprmalprv llllflhlll lgcrshplgg aglaselpgi qelldrlrdr vselqaertd
61 leplrqdrgl teaweareaa ptgvlgprss ifqvlrgirs pktmrdsgcf grrldrigsl
121 sglgcnvlrr y.

Detailed Disclosure of Invention

The present invention is based at least partly on the discovery that brain natriuretic peptides (BNP) and atrial natriuretic peptides (ANP) are produced in high levels by human bone marrow stromal cells (BMSCs) *in vitro*.

The present invention pertains to a method for treating deficits caused by focal or generalized edema associated with injury to organs or organ systems, such as the central nervous system, heart, liver and kidney. According to the method of the present invention, edema associated deficits are treated by the administration of cells that produce natriuretic peptides, such as BMSCs. Where BMSCs are utilized, the cells are preferably conditioned with retinoic acid and nerve growth factor before, during, or after administration to the patient, in order to increase the cells' production of natriuretic peptide. However, conditioning of BMSCs with retinoic acid and nerve growth factor is not necessary since BMSCs produce BNP *in vivo*.

The natriuretic peptides delivered by the transplanted cells increase excretion of sodium and water within the patient, mimicking the effects of direct infusion of the peptides. For example, the method of the present invention can be used to treat neurological deficits of the central nervous system that result from stroke, trauma, toxins, and other nervous system insults. Administration of natriuretic peptide producing cells into the brain results in decreased brain edema at the penumbra zone of injury, facilitating a more rapid recovery. Thus, infusion of natriuretic peptide producing cells, such as BMSC, systemically or intracerebrally is a novel way to treat any acute injury to brain, spinal cord or peripheral organs that results in edema.

ANPs do not cross the blood-brain-barrier, but BMSCs infused systemically do penetrate preferentially at the zone of the infarct or injury. BMSCs have also been shown to localize at the ischemic heart following systemic intravenous administration.

In another aspect, the present invention concerns pharmaceutical compositions comprising bone marrow stromal cells and effective amounts of retinoic acid and nerve growth factor to induce the bone marrow stromal cells to increase production of natriuretic peptides. The pharmaceutical composition can be adapted for various forms of parenteral administration, such as intravenous or intracerebral routes. Administration can be continuous or at distinct intervals as can be determined by a person skilled in the art.

The pharmaceutical compositions of the subject invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Formulations are described in a number of sources which are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Sciences* (Martin EW [1995] Easton Pennsylavania, Mack Publishing Company, 19th ed.) describes formulations which can be used in connection with the subject invention. Formulations suitable for parenteral administration include, for example, aqueous sterile injection solutions, which may contain antioxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the condition of the sterile liquid carrier, for example, water for injections, prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powder, granules, tablets, *etc.* It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the subject invention can include other agents conventional in the art having regard to the type of formulation in question.

In another aspect, the present invention provides a method for producing natriuretic peptides comprising culturing bone marrow stromal cells and isolating the natriuretic peptides from the bone marrow stromal cells. Peptide isolation methods known in the art can be utilized. For example, antibodies specific for natriuretic peptide can be used to isolate the peptides from culture. Preferably, the bone marrow stromal cells are cultured in the presence of retinoic acid and nerve growth factor, thereby inducing the bone marrow stromal cells to increase production of the natriuretic peptides by the bone marrow stromal cells. According to one embodiment of the present invention, bone marrow stromal cells are conditioned for about two to about four days *in vitro* with retinoic acid (0.5 μM) and NGF (100 ng/μL). After the cultures reach confluency, the cells can be lifted by incubation with trypsin (0.25%) and 1 mM EDTA at 37°C for 3–4 min. The BMSC cells are suspended in phosphate buffered saline (PBS) and infused into the systemic circulation (total of about 1-3 million cells).

A wide variety of media, salts, media supplements, and products for media formulation can be utilized to culture the BMSCs in the presence of retinoic acid and nerve growth factor, thereby conditioning the cells to increase production of natriuretic peptides according to the method of the present invention. Examples of these substances include, but are not limited to, carrier and transport proteins (*e.g.*, albumin), biological detergents (*e.g.*, to protect cells from shear forces and mechanical injury), biological buffers, growth factors, hormones, hydrosylates, lipids (*e.g.*, cholesterol), lipid carriers, essential and non-essential amino acids, vitamins, sera (*e.g.*, bovine, equine, human, chicken, goat, porcine, rabbit, sheep), serum replacements, antibiotics, antimycotics, and attachment factors. These substances can be present in various classic and/or commercially available media, which can also be utilized with the subject invention. Examples of such media include, but are not limited to, Ames' Medium, Basal Medium Eagle (BME), Click's Medium, Dulbecco's Modified Eagle's Medium (DMEM), DMEM/Nutrient Mixture F12 Ham, Fischer's Medium, Minimum Essential Medium Eagle (MEM), Nutrient Mixtures (Ham's), Waymouth Medium, and William's Medium E.

According to methods of the present invention, natriuretic peptide producing cells can be administered as cell therapy to alleviate the symptoms of a wide variety of disease states and pathological conditions associated with edema, in various stages of pathological development. Treatment with natriuretic peptide producing cells is intended to include prophylactic intervention to prevent onset of the symptoms associated with the particular edema-associated deficit, as well as intervention to alleviate or eliminate symptoms of an edema-associated deficit that are being presented by the patient at the time of administration of the cells. For example, natriuretic peptide producing cells can be used to treat acute disorders (*e.g.*, stroke or myocardial infarction), and administered acutely, subacutely, or in the chronic state. Similarly, the natriuretic peptide producing cells can be used to treat chronic disorders (*e.g.*, Parkinson's disease), and administered preventatively and/or prophylactically, early in the disease state, in moderate disease states, or in severe disease states.

The following pathological conditions are examples of diseases or conditions that cause edemic injury which may be treated in accordance with the present invention:

cerebrovascular accidents (also referred to as stroke), brain trauma, and spinal cord injuries. The following are examples of deficits associated with or induced by edemic injury: paralysis, loss of sensation, inability to speak or understand language, loss of consciousness and coma.

Preferably, treatment will substantially correct an edema-induced deficit. However, that may not always be possible. Thus, treatment according to the present invention, and with the cells and pharmaceutical compositions of the invention, may lead to improvement in function without complete correction.

The number of cells to be used will vary depending on the nature and extent of the edemic tissue. Typically, the number of cells used in transplantation will be in the range of about one hundred thousand to several million. One or more transplants can be carried out to further improve function.

Methods for transplantation of cells into human and non-human mammals are known to those skilled in the art and described in the scientific literature. The terms "transplantation", "administration", and "implantation" are used herein interchangeably and include the transplantation of cells which have been grown *in vitro*, and may have been genetically modified to produce natriuretic peptides, as well as the transplantation of material extracted from another organism. Natriuretic peptide producing cells can be transplanted by means of microsyringe infusion of a known quantity of cells in the target area. The phrase "intracerebral transplantation" as used herein includes transplantation into any portion of the brain, and is not restricted to the front and/or larger portion of the brain.

The natriuretic peptide producing cells can be administered as autografts, syngeneic grafts, allografts, and xenografts, for example. As used herein, the term "graft" refers to one or more cells intended for implantation within a human or other animal. Hence, the graft can be a cellular or tissue graft, for example.

The natriuretic peptide producing cells can be administered in an open manner to the target anatomical site, as in the heart during open heart surgery, or in the brain during stereotactic surgery, or by systemic procedures such as intravascular interventional methods using catheters going to the blood supply of the specific organs, or by other interventional methods known in the art.

Mammalian species which benefit from the disclosed methods of treatment include, and are not limited to, apes, chimpanzees, orangutans, humans, monkeys; domesticated animals (*e.g.*, pets) such as dogs, cats, guinea pigs, hamsters, Vietnamese pot-bellied pigs, rabbits, and ferrets; domesticated farm animals such as cows, buffalo, bison, horses, donkey, swine, sheep, and goats; exotic animals typically found in zoos, such as bear, lions, tigers, panthers, elephants, hippopotamus, rhinoceros, giraffes, antelopes, sloth, gazelles, zebras, wildebeests, prairie dogs, koala bears, kangaroo, opossums, raccoons, pandas, hyena, seals, sea lions, elephant seals, otters, porpoises, dolphins, and whales. As used herein, the term "patient" is intended to include such human and non-human mammalian species. The cells used in the subject invention can be any human or non-human mammalian cells.

Combinations of cell types can be co-administered to enhance therapeutic potential. For example, a trophic factor-producing cell line can be co-administered with a neuronal cell line that has been genetically modified to produce a natriuretic peptide, such as BNP or ANP. Sertoli cells can be co-administered with natriuretic peptide producing cells of a species different from that of the recipient, such that the Sertoli cells provide local immunosuppression of the xenograft.

Methods for transplanting various nerve tissues as allografts and xenografts have been described previously (Freeman T.B. *et al.*, *Progress in Brain Research*, 1988, Chapter 61, Elsevier Science Publishers, 78:473-477; Freeman T.B. *et al.*, *Parkinson's Disease: Advances in Neurology*, 2001, Chapter 46, Lippincott Williams & Wilkins, 86:435-445; Freeman T.B. *et al.*, *Annals of Neurology*, 1995, 38(3):379-387; Freeman T.B. *et al.*, Progress in Brain Research, 2000, Chapter 18, Elsevier, Amsterdam, 127:405-411; Olanow C.W. *et al. The Basal Ganglia and New Surgical Approaches for Parkinson's Disease, Advances in Neurology*, 1997, 74:249-269; Bjorklund *et al.*, Neural Grafting in the Mammalian CNS, 1985, p. 709, Elsevier, Amsterdam; Das G.D., "Neural Grafting in the Mammalian CNS, 1985, Chapter 3, p. 23-30, Elsevier, Amsterdam). These procedures include intraparenchymal transplantation, *i.e.*, within the host brain tissue (as compared to outside the brain or extraparenchymal transplantation) achieved by injection or deposition of tissue within the host brain so as to be opposed to the brain parenchyma at the time of transplantation.

Methods for intraparenchymal transplantation include, for example: (i) injecting the donor cells within the host brain parenchyma (*e.g.*, stereotactically, using image guidance, and/or with a catheter attached to a pump, such as a MEDTRONIC system); and (ii) preparing a cavity by surgical means to expose the host brain parenchyma and then depositing the graft into the cavity. Such methods provide parenchymal apposition between the graft and host brain tissue at the time of grafting, and both facilitate anatomical integration between the graft and host brain tissue.

Alternatively, the graft can be placed into the cerebral spinal fluid (CSF), either by open surgical injection, intraventricularly via a needle or ventricular reservoir, into the lumbar subarachnoid space using a lumbar puncture, or into any CSF site using a pump and a catheter (*e.g.*, MEDTRONIC). These methods would lend themselves to repeated administration over time, to the CSF or to the brain. Grafting to the ventricle may be accomplished by injection of the donor cells or by growing the cells in a substrate such as 3% collagen to form a plug of solid tissue which may then be implanted into the ventricle to prevent dislocation of the graft. For subdural grafting, the cells may be injected around the surface of the brain after making a slit in the dura. Injections into selected regions of the host brain can be made by drilling a hole and piercing the dura to permit the needle of a microsyringe to be inserted. The microsyringe can be mounted in a stereotactic frame and three-dimensional stereotactic coordinates are selected for placing the needle into the desired location of the brain or spinal cord. Image guidance methods can also be utilized. The cells of the subject invention can also be introduced into the putamen, caudate nucleus, pallidum, nucleus basalis, hippocampus, cortex, cerebellum, subcortical white matter, other regions of the brain, as well as the spinal cord.

The methods of the subject invention also contemplate the administration of cells that have been genetically modified to produce a natriuretic peptide, such as BNP or ANP. Such genetically modified cells can be administered alone or in combinations with different types of cells. Thus, genetically modified cells of the invention can be co-administered with other cells, which can include genetically modified cells or non-genetically modified cells. Genetically modified cells may serve to support the survival and function of the co-administered cells, for example.

SEQ ID NOs. 1 and 3 are genes encoding human BNP and human ANP, respectively. SEQ ID NOs. 10 and 11 are the coding sequences of human BNP and human ANP, respectively. In specific embodiments, the cells of the invention are genetically modified with nucleotide sequences encoding human BNP and/or human ANP (SEQ ID NOs. 2 and 4, respectively), such as the nucleotide sequences of SEQ ID NO: 1, 3, 10, or 11. In other embodiments, nucleotide sequences encoding non-human mammalian homologs of natriuretic peptides are used, such as nucleotide sequences encoding SEQ ID NO:6, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, and SEQ ID NO:21. Exemplified nucleotide sequences encoding non-human mammalian homologs of natriuretic peptides are SEQ ID NO:5, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17.

The term "genetic modification" as used herein refers to the stable or transient alteration of the genotype of a cell of the subject invention by intentional introduction of exogenous nucleic acids by any means known in the art (including for example, direct transmission of a polynucleotide sequence from a cell or virus particle, transmission of infective virus particles, and transmission by any known polynucleotide-bearing substance) resulting in a permanent or temporary alteration of genotype. The nucleic acids may be synthetic, or naturally derived, and may contain genes, portions of genes, or other useful polynucleotides in addition to those encoding natriuretic peptides. A translation initiation codon can be inserted as necessary, making methionine the first amino acid in the sequence. The term "genetic modification" is not intended to include naturally occurring alterations such as that which occurs through natural viral activity, natural genetic recombination, or the like. The genetic modification may confer the ability to produce natriuretic peptides wherein the cell did not previously have the capability, or the modification may increase the amount of natriuretic peptides produced by the cell, *e.g.*, through increased expression.

Exogenous nucleic acids can be introduced into a cell by viral vectors (retrovirus, modified herpes virus, herpes virus, adenovirus, adeno-associated virus, and the like) or direct DNA transfection (lipofection, calcium phosphate transfection, DEAE-dextran, electroporation, and the like), for example.

Preferably, the exogenous nucleotide sequence encoding a natriuretic peptide is operably linked to a promoter sequence that permits expression of the nucleotide sequence in a desired tissue within the patient. The promoters can be inducible or tissue specific as necessary. Means for inducible regulation of human BNP are known (Quan H. *et al.*, *Am. J. Physiol. Heart Circ. Physiol.*, 2001, 280:H368-H376). Examples of other promoters that can be utilized to express nucleotides encoding natriuretic peptides within a mammalian cell, such as a human cell, include cytomegalovirus promoter (Boshart *et al.*, *Cell*, 1985, 41:521-530) and SV40 promotor (Subramani *et al.*, *Mol. Cell. Biol.*, 1981, 1:854-864).

The term "operably-linked" is used herein to refer to an arrangement of flanking sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking sequence operably-linked to a coding sequence may be capable of effecting the replication, transcription and/or translation of the coding sequence. For example, a coding sequence is operably-linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence, and the promoter sequence can still be considered "operably-linked" to the coding sequence. Each nucleotide sequence coding for a natriuretic peptide will typically have its own operably-linked promoter sequence.

The nucleotide sequences encoding natriuretic peptides used in the subject invention include "homologous" or "modified" nucleotide sequences. Modified nucleic acid sequences will be understood to mean any nucleotide sequence obtained by mutagenesis according to techniques well known to persons skilled in the art, and exhibiting modifications in relation to the normal sequences. For example, mutations in the regulatory and/or promoter sequences for the expression of a polypeptide that result in a modification of the level of expression of a polypeptide according to the invention provide for a "modified nucleotide sequence". Likewise, substitutions, deletions, or additions of nucleic acid to the polynucleotides of the invention provide for "homologous" or "modified" nucleotide sequences. In various embodiments,

"homologous" or "modified" nucleic acid sequences have substantially the same biological or serological activity as the native (naturally occurring) natriuretic peptide. A "homologous" or "modified" nucleotide sequence will also be understood to mean a splice variant of the polynucleotides of the instant invention or any nucleotide sequence encoding a "modified polypeptide" as defined below.

A homologous nucleotide sequence, for the purposes of the present invention, encompasses a nucleotide sequence having a percentage identity with the bases of the nucleotide sequences of between at least (or at least about) 20.00% to 99.99% (inclusive). The aforementioned range of percent identity is to be taken as including, and providing written description and support for, any fractional percentage, in intervals of 0.01%, between 20.00% and 99.99%. These percentages are purely statistical and differences between two nucleic acid sequences can be distributed randomly and over the entire sequence length.

In various embodiments, homologous sequences exhibiting a percentage identity with the bases of the nucleotide sequences of the present invention can have 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent identity with the polynucleotide sequences of the instant invention. Homologous nucleotide and amino acid sequences include, but are not limited to, mammalian homologues of the human natriuretic peptide sequences. Homologous nucleotide sequences, when expressed, exhibit substantially the same biological activity (*e.g.*, anti-edema effects) as the native sequence, as can be determined *in vitro* or *in vivo*.

Both protein and nucleic acid sequence homologies may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman *Proc. Natl. Acad. Sci. USA*, 1988, 85(8):2444-2448; Altschul *et al. J. Mol. Biol.*, 1990, 215(3):403-410; Thompson *et al. Nucleic Acids Res.*, 1994, 22(2):4673-4680; Higgins *et al. Methods Enzymol.*, 1996, 266:383-402; Altschul *et al. J. Mol. Biol.*, 1990, 215(3):403-410; Altschul *et al. Nature Genetics*, 1993, 3:266-272).

In another embodiment, the natriuretic peptide producing cells are derived from transgenic animals, and thus are in a sense already genetically modified. There are several methods presently used for generating transgenic animals. A typical technique is direct microinjection of DNA into single-celled fertilized eggs. Other methods include retro-viral-mediated transfer, or gene transfer in embryonic stem cells. These techniques and others are detailed by Hogan *et al.* in Manipulating the Mouse Embryo, A Laboratory Manual (Cold Spring Harbor Laboratory Ed., 1986).

In addition to the objective of delivering natriuretic peptides to a patient in need thereof, the genetically modified cells of the subject invention can be administered to a patient for cell/gene therapy, *e.g.*, for *in vivo* delivery of various biomolecules, such as the trophic factors described above. Alternatively, the genetically modified cells can be used as biological "factories" to provide the product of the exogenous DNA (*e.g.*, natriuretic peptide) and/or the natural product of the modified cells *in vitro*, or *in vivo* within an animal.

Genetically modified cells can be stem cells or non-stem cells. Thus, non-stem cells (*e.g.*, specialized or mature cells, and their precursor or progenitor cells) can be genetically modified to produce natriuretic peptides. Genetic modification with nucleotide sequences encoding natriuretic peptides, such as BNP and ANP, can be used to produce cells that have therapeutic potential for injuries related to multiple or specific organs. For example, cardiomyocytes or their progenitors can be genetically modified to target heart injury; hepatocytes or their progenitors can be genetically modified to target liver injury; and islet cells or their progenitors can be genetically modified to target diabetes. Any of the over 200 cells naturally occurring in mammals can be genetically modified to provide cellular delivery of one or more natriuretic peptides. Examples of cells that can be genetically modified include, but are not limited to, bone marrow cells, stem cells, neural cells, Sertoli cells, fat cells, placental cells, fibroblasts, and umbilical cord blood cells.

According to the methods of the present invention, whether genetically modified or non-genetically modified, the natriuretic peptide producing cells can be co-administered with other therapeutic agents useful in treating defects, trauma, or diseases, such as growth factors, antibiotics, or neurotransmitters.

Cells can be genetically modified to include regulators, inducible promoters, tissue-specific promoters, on-off genes, or suicide genes. Genetically modified cell lines can include more than one genetic construct.

Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989) and in Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons, New York (1988), and in Watson *et al.*, Recombinant DNA, Scientific American Books, New York and in Birren *et al.* (eds) Genome Analysis: A Laboratory Manual Series, Vols. 1-4 Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in PCR Protocols: A Guide To Methods And Applications, Academic Press, San Diego, Calif. (1990). In-situ (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni *et al.*, *Blood*, 1996, 87:3822.)

Materials and Methods

Human Bone Marrow Stromal Cell Cultures. Human bone marrow cells are not easy to obtain for basic research since they are a scarce resource for treatment of certain leukemias. We used an easily accessible alternative source of bone marrow stromal cells (BMSC). The method has been described in detail (Song, S. and Sanchez-Ramos, J., "Preparation of Neural Progenitors from Bone Marrow and Umbilical Cord Blood" in Protocols for Neural Stem Cell Methods, Zigova, T. *et al.*, Eds., 2002, pp.79-88). Bone marrow from volunteer donors were routinely passed through a nylon filter to remove debris from the sample prior to preparation for bone marrow replacement. The nylon filter retains bony chips with adherent bone marrow cells, fatty tissue and other debris. The nylon filters were washed with sterile saline solution five times 4 of 19 and centrifuged to remove bone chips. The cells in the supernatant were then processed as described and plated in uncoated polyethylene culture flasks in Dulbecco's minimal

essential medium (DMEM; Gibco/BRL, Inc) and fetal bovine serum 10% (FBS; Hyclone). When adherent cells reached 60-70% confluence, they were raised from the plates, resuspended and re-plated in new flasks.

Five human bone marrow donors provided the nylon filters that contain bone marrow debris from which BMSC were subsequently prepared.

1. Male 34 y/o BMSC; assays performed at passage 3 and passage 5
2. Male 50 y/o BMSC; assays performed at passage 1 and passage 3
3. Male 59 y/o BMSC; assays performed at passage 1 and passage 3
4. Female 44 y/o BMSC; assays performed at passage 11 and passage 13
5. Female 34 y/0 BMSC: immunocytochemical assay at passage 4.

The cultures were incubated under three distinct media conditions. Condition A: DMEM+FBS10% for 6 days. Condition B: DMEM+FBS10% for two days followed by Nerve Growth Factor (NGF 100 ng/mL) + all-trans retinoic acid (RA; 0.5 μg/mL) for four days. Condition C: N2 + 20 ng/mL of Epidermal Growth Factor (EGF; GIBCO) + 10 ng/mL of basic Fibroblast Growth Factor (bFGF; GIBCO) for two days followed by NGF+RA for four days.

Immunocytochemistry and estimates of cell density. Cells from a 34 y/o female were plated in 35 mm multi-well plates at passage four with a density of approximately 7.5 X 10^5 cells per well. After a total of six days *in vitro* (under conditions described above), the cultures were fixed in 5% phosphate-buffered paraformaldehyde for 30 minutes, washed three times with phosphate buffered saline and incubated with rabbit antiserum to human BNP-32 (PENINSULA LABORATORIES, Inc.; Belmont, CA) at a dilution of 1:200 for 24 hrs at 4°C. FITC conjugated anti-rabbit antibodies (CHEMICON INTERNATIONAL Inc.) were used to visualize the BNP immunoreactive cells. Cultures were also incubated without primary antibody to control for non-specific staining by the anti-rabbit antibody, DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; MOLECULAR PROBES, Eugene OR) staining of nuclei was used to visualize total number of cells per visual field. Estimates of cell density were based on counts of DAPI-stained nuclei in 10 visual fields per culture dish.

DNA Microarray Analysis. Total RNA was isolated from cell cultures using the RNA STAT-60 kit according to the protocol recommended by the manufacturer (TEL-

TEST "B," Inc. Friendswood, TX 77546). Following RNA isolation, its OD density was measured at 260 nm, and stored at 280°C. Integrity was tested on 1% nondenaturing Seakem LE agarose gel (FMC BIO-PRODUCTS, Rockland, ME). As previously described the samples were probed on the human genome U95A array (HGU95A) from AFFYMETRIX, Inc (Sanchez-Ramos *et al.*, 2001). The single array represents;12,500 sequences. The experiment was done at the Functional Genomics Core, Microarray Facility, at the H. Lee Moffitt Cancer Center and Research Institute using GeneChip Fluidics station 400, a Gene-Chip Hybridization oven, and an HP GeneArray scanner. Analysis of the GeneChip microarray hybridization pattern was performed using GeneChip Analysis Suite 4.0 software.

RT-PCR. Both RT and PCR were performed with PERKIN ELMER's GENEAMP RNA PCR kit. RT was performed using total RNA (1 μg) and random hexamers as a primer. PCR was done using Human BNP (GeneBank accn.# M31776) primers (Forward: nt. 1320-1341 and Reverse: nt. 1597-1578) on a PE 9700 thermocycler (PERKIN ELMER, Foster City, CA.).

Real Time Quantitative RT-PCR. Real-time quantitative RT-PCR analyses were performed using the ABI PRISM 7700 Sequence Detection System (APPLIED BIOSYSTEMS, Foster City, CA). Primers and probes for human BNP were designed and synthesized by APPLIED BIOSYSTEMS (Foster City, CA) from GenBank accession number M31776. The probe hBNP-exonM2 (5'-CGCTGCTCCTGTAAC-3') (SEQ ID NO:7) was labeled with 6-carboxyfluorescein as the reporter and a minor groove binder moiety (MGB) on the 3'-end. The forward primer was 5'-GCCTCGGACTTGGAAACGT-3' (SEQ ID NO:8) and the reverse primer was 5'-TGCAGCTCCGACAGTTTGC-3' (SEQ ID NO:9).

Sample RNA (5 μl of 50 ng/μl, 5 μl of 400 ng/μl) was transcribed into cDNA using Superscript II-reverse transcriptase (LIFE TECHNOLOGIES, Inc.) and 250 ng of random hexamers (LIFE TECHNOLOGIES, Inc.) following the manufacturer's directions in a final volume of 20 μl. For the construction of standard curves RNA was diluted in DEPC-treated water (AMBION, Austin, TX) before reverse transcription.

PCR was carried out with the TAQMAN Universal PCR Master Mix (APPLIED BIOSYSTEMS) using 3 μl of diluted cDNA, 200 nM probe, and 900 nM in a 30-μl final

reaction mixture. After a 2-min incubation at 50°C, AMPLITAQ GOLD was activated by a 10-min incubation at 95°C. Each of the 40 PCR cycles consisted of 15 s of denaturation at 95°C and hybridization of probe and primers for 1 min at 60°C. Amounts of human BNP and 18s RNAs were calculated using linear regression analysis from an external standard curve.

Radioimmunoassay for Brain Natriuretic Peptide (BNP). The assay is based upon the competition of ^{125}I-BNP and BNP of the sample binding to the limited quantity of specific antibodies for BNP. Cells and media were diluted with 100% ethanol (1:2 dilution), vortexed, and allowed to stand at 4°C for 30 minutes. The samples were centrifuged and the supernatants were air-dried and stored at –80°C until time of assay. The samples were incubated with rabbit anti-BNP antibody and normal rabbit serum for 24 hours at 4°C. Then, the samples were incubated with ^{125}I-BNP (13,000 cpm) for 24 hours at 4°C. Goat antirabbit IgG serum were added to the samples and incubated for 2 hours at room temperature. The samples were centrifuged at 3000g for 20 minutes at 4°C. The radioactivity was counted with a gamma-counter. A standard curve was constructed by measuring the amount of ^{125}I-BNP bound to known concentrations of BNP.

Following is an example which illustrates materials, methods, and procedures for practicing the invention. The example is illustrative and should not be construed as limiting.

Example—Production of Brain Natriuretic peptide (BNP) by Bone Marrow

BMSC cultures were incubated with a) DMEM and FCS 10% for six days or with b) DMEM and FCS 10% for two days with a change of medium to retinoic acid and NGF for four days or with c) N2 + EGF + bFGF for two days followed by NGF+RA for four days. Examination of the cultures under phase contrast microscopy revealed a change in morphology when growth factors were added (See Figure 1). The untreated cultures (condition A) remained predominantly flat and assumed a sheet-like arrangement of cells (Figure 1, row A). The cultures treated with growth factors conditions B or C (Figure 1, row B) assumed a fibroblastic morphology. Under fluorescence microscopy all sets of cultures were immunoreactive for BNP. However, in condition A (no RA or growth

factors), a small number of large flat cells were not BNP immunoreactive. In cultures treated with growth factors (Figure 1, row B), most of the fibroblastic cells were immunoreactive for BNP. The mean density of cells in the untreated cultures (estimated by counting DAPI-stained nuclei) was 6.2 X 10^5 cells per culture dish (with a range of 4.0 to 7.5 X 10^5); the mean density of cells in the growth factor treated cultures (condition B) was 4.5 X 10^5 cells per culture dish (range of 3.2 to 5.4 x 10^6). Although there was a slight trend towards decreased numbers of cells as they changed morphology following the addition of growth factors, the difference in cell density under the two cell culture conditions after six days *in vitro* was not significant. The studies of BNP protein expression were carried out after six days *in vitro*. The cell densities were approximately 6.2 x 10^5 in condition A and 4.5 X 10^5 in conditions B and C (RA and growth factor treated cultures). For studies of gene expression using RT-PCR, cultures were grown in T-75 culture flasks at cell densities of approximately 3 X 10^6 cells per flask. In a screening study of gene expression by BMSC undergoing differentiation, total RNA was extracted, and cDNA was prepared for hybrization with the oligonucleotides contained on the human U95A array (AFFYMETRIX, Inc.). Preliminary evidence revealed that transcripts for BNP were highly upregulated in the RA+NGF treated cultures in two microarray experiments using two different donor samples. Since this was designed to be quick survey of the transcriptional profile of these cultures, there were insufficient samples and experiments to perform statistical or cluster analyses. Review of the microarray data for changes in ANP and other regulators of sodium homeostasis, including rennin and aldosterone, was performed and none of these regulators were increased in RA-induced BMSC differentiation.

To validate the presence of BNP mRNA in the cell cultures, RT-PCR and quantitative RT-PCR was performed. In addition, a sensitive radioimmunoassay method was employed to quantify the levels of BNP protein produced within the cells and secreted into the medium.

Figure 2 shows results from a single RT-PCR experiment run to confirm the presence of BNP in the BMSCs. The band in the RA+NGF column suggests a greater intensity than in the DMEM control sample, though this example was not designed to be quantitative. Also shown in the same gel are bands for Musashi-1 and nestin mRNA,

both of which are considered to be markers of neural progenitors (Kaneko, Y. *et al.*, *Developmental Neuroscience*, 2000, 22:139-153; Lendahl, U. *et al.*, *Cell*, 1990, 60:585-595). There have been a number of investigations that have reported neural protein markers expressed by bone marrow stromal cells, but it remains uncertain as to whether these cells differentiate into functional neurons (Sanchez-Ramos, J.R. *J. Neurosci. Res.*, 2002, 69:880-893).

Figure 3 shows the results obtained by applying real-time PCR to the RNA extracted from the cultures. Quantitative data were derived from two experiments using BMSC samples from different donors. BNP mRNA was increased 8 fold under condition B (RA+NGF) and 11 fold under condition C (EGF, bFGF, RA, NGF) compared to condition A (no growth factors). Insufficient number of samples were analyzed to perform a statistical analysis. In order to determine whether expression of BNP protein was increased, and to gather sufficient samples to perform statistical analyses, a sensitive radioimmunoassay was applied to 8 samples of BMSC incubated under three different conditions.

Figure 4 shows summary data using BMSC from 4 different marrow samples (donated by subjects 1 to 4). BMSC from each of the donors was assayed at two different passages (n = 8). BNP levels were measured in both the cells and supernatant in each of the three culture conditions. The levels of BNP measured within the cells did not change for any of the culture conditions and remained around 20 pmol/L. However, the mean levels of BNP released into the media was significantly increased (3-4 fold) by addition of retinoic acid and growth factors ($p<0.001$ in condition B and $p<0.01$ in condition C). The mean supernatant BNP level was 75 (SD=16, n=8) in cultures incubated with DMEM+FCS for 6 days (Condition A). In cultures incubated for 2 days with DMEM+FBS followed by 4 days with RA+NGF (Condition B), the mean BNP level was 317 (SD=100, n=8). When cultures were incubated for 2 days with EGF+FGF followed by 4 days with RA+NGF (Condition C), the mean levels of BNP were 276 (SD=77, n=8). 2-way ANOVA demonstrated that culture conditions as well as cell/supernatant contributed significantly to the variance ($p<0.05$ for culture condition and $p<0.001$ for supernatant). t-tests with Bonferroni correction show significant differences between cells and supernatant for condition B ($p< 0.001$) and for condition C ($p< 0.01$) but not for

condition A ($p>0.05$). Even though the number of cells per dish in growth factor-treated cultures tended to be lower than DMEM+FBS treated cultures after 6 days *in vitro*, the average concentration of BNP released into the medium by the cultures incubated with RA and growth factors was significantly higher.

This is the first report that bone marrow is capable of producing significant levels of BNP. The mean levels of BNP secreted by BMSC with and without addition of growth factors (mean of 75 pmol/L in DMEM and 317 pmol/L in growth factor-treated cultures) far exceeds the BNP found circulating in the serum and CSF of normal human subjects. The mean serum BNP level in 124 healthy subjects was 1.8 +/- 1.0 pmol/L (SD), with a range of 0.6-5.5 pmol/L (Jensen *et al.*, *Scand J Clin Lab Invest*, 1997, 57:529-540). In eight patients with congestive heart failure, the median BNP level was 30.5 pmol/L, with a range of 3.9-65.3. The mean BNP concentration in human cerebrospinal fluid collected from fifteen patients without neurological disorders has been reported to be 0.27 +/- 0.10, pmol/L (mean +/- S.D (Kaneko *et al.*, *Brain Res*, 1993, 612:104-109).

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.